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Phenolic compounds and antioxidant activity in red fruits produced in organic farming

Raquel P. F. Guiné[1,2*], Susana M. A. Soutinho[1], F. J. Gonçalves[1,2]

[1]*Dep. Indústrias Alimentares – Escola Superior Agrária de Viseu, Viseu, Portugal*
[2]*CI&DETS – Instituto Politécnico de Viseu, Viseu, Portugal*

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Summary

In this work were studied three red fruits (raspberry, gooseberry and blueberry) produced in organic mode, to evaluate the variations in the content of phenolic compounds and antioxidant capacity along maturation. The phenols were extracted from the fruits with two solvents (methanol and acetone) and were quantified by the Folin-Ciocalteu method. The antioxidant activity was determined with two methods (HPPH and ABTS). Furthermore, HPLC was used to identify and quantify some phenolic compounds present in the fruits analyzed. The results showed that the total phenolic compounds in all fruits decreased along maturation, either in the methanol or acetone extracts (23 % and 20 % reduction, on average, for methanol and acetone extracts, respectively), although in methanol extracts the levels of phenolic compounds were always higher (0.54 and 0.21 mg GAE/g). The blueberry showed higher level of total phenolics in methanol extract (average 0.67 mg GAE/g), while in the acetone extract it was gooseberry (average 0.31 mg GAE/g). At the end of maturation, all fruits studied had similar values of antioxidant capacity as determined by DPPH method (0.52 mmol Trolox/g). For the ABTS method, blueberries showed higher values of antioxidant activity (6.01 mmol Trolox/g against 3.01 and 2.66 mmol Trolox/g, for raspberry and gooseberry, respectively). Furthermore, the HPLC analysis allowed to identify monomeric anthocyanins and phenolic acids in the three fruits studied.

Keywords: antioxidant activity, blueberry, gooseberry, organic farming, phenolic compounds, raspberry

Introduction

Organic farming or biological production mode differs from other production methods for its balanced and constructive action in agricultural systems. With the increase in intensive agriculture, undesirable changes were being observed in ecosystems with irreparable damage being caused to the natural equilibrium. This is the reason for the increasing interest in organic farming as an environment friendly agricultural production method. Organic farming is distinguished from other production systems because it excludes almost all synthetic chemicals, uses cultural rotations, crop residues, animal manure, and all organic waste from the farm (Soutinho et al., 2013a, 2013b).

Currently there has been increasing interest in organically produced food products. In parallel, has increased the demand for fruits, given their high content of biologically active phytochemical compounds, capable of delaying the onset of diseases, contributing to the health and welfare of humans (Soutinho et al., 2013a, 2013b).

The red fruits are foods quite rich in essential micronutrients and bioactive compounds and should be part of a balanced diet. Studies have shown that in the group of small fruits, which include raspberry, blueberry and blackcurrant, each species has distinctive and specific levels of phenolic compounds, consisting mainly of anthocyanins, flavonols, proanthocyanidins, and phenolic acids, catechins and isoflavones, compounds known for their antioxidant capacity (Chiang et al., 2013; Gevrenova et al., 2013; Lee et al., 2012; Nour et al., 2013; You et al., 2011).

Blueberry (*Vaccinium corymbosum*) is known to contain significant levels of phenolic compounds, including anthocyanins, flavonoids and procyanidins that have high biological activity, providing health benefits as antioxidants (Koca and Karadeniz, 2009). Raspberry (*Rubus Idaeus*) is also quite rich in phenolic compounds especially in anthocyanins , cyanidin-3-sophoroside, cyanidin-3-(2(G)-glucosylrutinoside), cyanidin-3-glucoside, cyanidin-3-rutinoside, pelargonidin-3-sophoroside, pelargonidin-3-(2(G)-glucosylrutinoside), and pelargonidin-3-glucoside (Mullen et al., 2002). The Gooseberry (*Phyllanthus distichus*) consists of small red berries that grow in clusters. It is particularly rich in vitamin C, especially the black and red currant. Acts effectively as an inhibitor of free radicals having

*Corresponding author: raquelguine@esav.ipv.pt

a wide range of antioxidant phytochemicals (Pantelidis et al., 2007).
In this work three red fruits, raspberry, gooseberry and blueberry, produced in organic farming, were characterized in relation to their phenolic composition and antioxidant activity along maturation. The objective of the work undertaken was to verify the evolution of the phenolic compounds and antioxidant activity along maturation, as well as to compare the different red fruits studied in order to identify those richer in pgenolic compounds. Furthermore, it was also intended to identify the individual phenolic compounds present in each sample by means of HPLC.

Materials and methods

Sampling

The fruits studied in this work were raspberry, gooseberry and blueberry, all produced in organic farming and provided by a local producer. The samples were harvested for analysis between the 3rd of June and 9th of July, along maturation and until the complete maturation stage. The sample size was over 100 fruits of each kind at each harvesting date, and they were chosen randomly from the plantation but looking for uniformity in size and maturation status.

Extraction procedure

The phenolic compounds present in the fruits were extracted according to an adaptation of the method proposed by Ferreira et al. (2002). Each sample consisted of 10 g, taken from a mass obtained from grinding several fruits, so as to be representative. The extraction of phenolic compounds was carried out through maceration and subsequent extraction three times with a solution of methanol:acetic acid (98:2) followed by three times with a solution acetone:water (60:40). All the extractions were made from the same sample, successively, for 1 hour each, with magnetic stirring at room temperature. This allowed obtaining the methanol extracts and acetone extracts, respectively.

Phenolic Content

The phenolic content of the different extracts was determined by Folin-Ciocalteu reagent (Singleton and Rossi, 1965). Each sample (0.125 mL) was added to 0.5 mL of deionized water and 0.125 mL of Folin-Ciocalteu reagent (Sigma). After 6 min, 1.25 mL of 7.5 % solution of sodium carbonate and 1.0 mL of deionized water were added. The mixture was left 90 min at room temperature in the dark and the absorbance at 760 nm was measured. A calibration curve was made with standard solutions of gallic acid. The results were expressed in equivalents of the standard used. All analyses were done in triplicate.

Antioxidant capacity

The antioxidant capacity was determined by the methods using the free radicals 2,2-Diphenyl-1-picrylhydrazyl (DPPH*) and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) ABTS, respectively described by Brand-Williams et al. (1995) and Miller et al. (1993). The results were based on the percentage of inhibition of each fruit, compared with a standard antioxidant (Trolox) in a dose–response curve being expressed in Trolox equivalents.
In the first method, to a tube were added 100 μL of sample and 2 mL of DPPH previously prepared and then was placed placed in a dark place at room temperature for 30 minutes. Finally absorbance was measured in a spectrophotometer at a wavelength of 515 nm. The analysis was performed in triplicate.
In the second method, in the tube 100 μL of sample were combined with 2 ml of ABTS, previously prepared, and again the samples were placed in the dark at room temperature, this time for 15 minutes. Finally the absorbance of the samples was read at a wavelength of 734 nm. Three repetitions were also carried out in each sample.

Total tannins

Total tannins (TT) were estimated according to Ribereau-Gayon and Stonestreet (1966). Briefly, the sample was diluted to 1/50 in water. 2.0 mL of the previous solution was added to 1.0 mL of water and 3.0 mL of 12 M HCl. The content was divided into two tubes. One of them was heated for 30 min in boiled water and cooled (tube A), while the last one stayed at room temperature (tube B). A 0.5 mL amount of 95 % ethyl alcohol was added. The absorbance was read at 550 nm for each tube, AbstA and AbstB. Total tannins (g/L) were calculated using the equation: $TT = 19.33 \times (AbstA - AbstB)$.

Total Anthocyanins

Total anthocyanins (TA) were determined using the SO_2 bleaching method [5]. Each sample (1 mL) was added to 1 mL of ethanol acidified by 0.1 % HCl and 20 mL of 2 % HCl solution. In one tube, 2 mL of

previous solution was added to 0.8 mL of water (t1). In another tube (t2) were mixed 2 mL of previous solution and 0.4 mL of HNaSO3 solution (15 % w/v). After 20 min at dark room temperature, the absorbance at 520 nm was measured. The TA were calculated using the equation TA = 875 × (abst1 - abst2), and results expressed as mg/L of malvidin equivalents (Soutinho et al., 2013b).
The determination of the percentage of the different forms of polymerization of the anthocyanins (monomeric, polymeric and co-pigmented) was done following the method proposed by (Mazza et al., 1999).

Fractionation of Phenolic Compounds

The fractionation of fruit phenolic compounds was based on the methodology proposed by Sun *et al*. (2002). The pH of phenolic extracts was adjusted at pH≈7.0. This solution was loaded into the column in a flow of less than 2 mL/min. Phenolic acids fractions were eluted with 50 mL of diluted pH 7.0 phosphate buffer (1/8, v/v). The column was than washed with distilled water and ethyl acetate. The fraction composed of anthocyanins and polymeric proanthocyanidins was removed from the column by elution with methanol acidified by 0.1 % of HCl.

HPLC Procedure

The individual phenolic acids and monomeric anthocyanins were analyzed using a HPLC Dionex Ultimate 3000 Chromatographic System (Sunnyvale, California, USA) equipped with a quaternary pump Model LPG-3400 A, an ACC-3000 auto sampler, having a thermostatted column compartment (adjusted to 30 ºC) and a multiple Wavelength Detector MWD-300. The column (250 x 4.6 mm, particle size 5 μm) was a C18 Acclaim® 120 (Dionex, Sunnyvale, California, USA) protected by a guard column of the same material.

Phenolic acids

The phosphate buffer fraction obtained by C18 sep-pak was used for chromatographic analysis of the phenolic acids. The solvents were (A) water/formic acid (95:5 v/v), and solvent (B) methanol. Analysis conditions were as follow: a linear gradient analysis for a total run time of 80 min was used as follows: starting from 5 % solvent B during 2 min, increase to 80 % solvent B over 68 min and then isocratic for 8 min, decreasing to 5 % solvent B over 2 min, and finally isocratic for 5 min. The sample volume injected was 40 μL, the flow rate was 1.0 mL/min, and the column temperature was maintained at 30 ºC during the run.
The quantification of the individual hydroxybenzoic and hydroxycinnamic acids was made by a calibration curve obtained with standard solutions of gallic acid. The results were expressed in equivalents of gallic acid. The chromatographic peaks of all phenolic acids were identified by comparing their retention times with the retention time of standard compounds.

Monomeric anthocyanins

The monomeric anthocyanins present in the sample solutions were analyzed by HPLC. The solvents were (A) 40 % formic acid, (B) pure acetonitrile and (C) bidistilled water. The initial conditions were 25 % A, 10 % B, and 65 % C, followed by a linear gradient from 10 to 30 % B, and 65 to 45 % C for 40 min, with a flow rate of 0.7 mL/min. The injection volume was 20 μL. The detection was made at 520 nm and a Chromeleon (version 6.8) software program (Sunnyvale, California, USA) was used.
The quantification of the individual anthocyanins was made by a calibration curve obtained with standard solutions of malvidin-3-glucoside. The anthocyanin structures were isolated and identified according to their UV-Vis spectrum (Dallas and Laureano, 1994).

Results and discussion

Fig. 1 shows the evolution of total phenolic compounds present in the extracts of methanol and acetone, expressed as gallic acid equivalents (GAE) per gram, along ripening for the different fruits studied. At the beginning of maturation (03/Jun) the gooseberry sample had a value of total phenolic compounds higher when compared to the other two fruits. It can be seen that along the ripening process, the content of phenolic compounds present in the extracts of methanol and acetone remained approximately constant for the raspberry and blueberry. On the contrary, in the case of gooseberry, and despite some oscillations, a trend toward a decrease in the phenolic content was observed. The amount of phenolic compounds present in the methanol extract was always higher than that of the acetone extract to all the fruits and in all of the harvest dates.

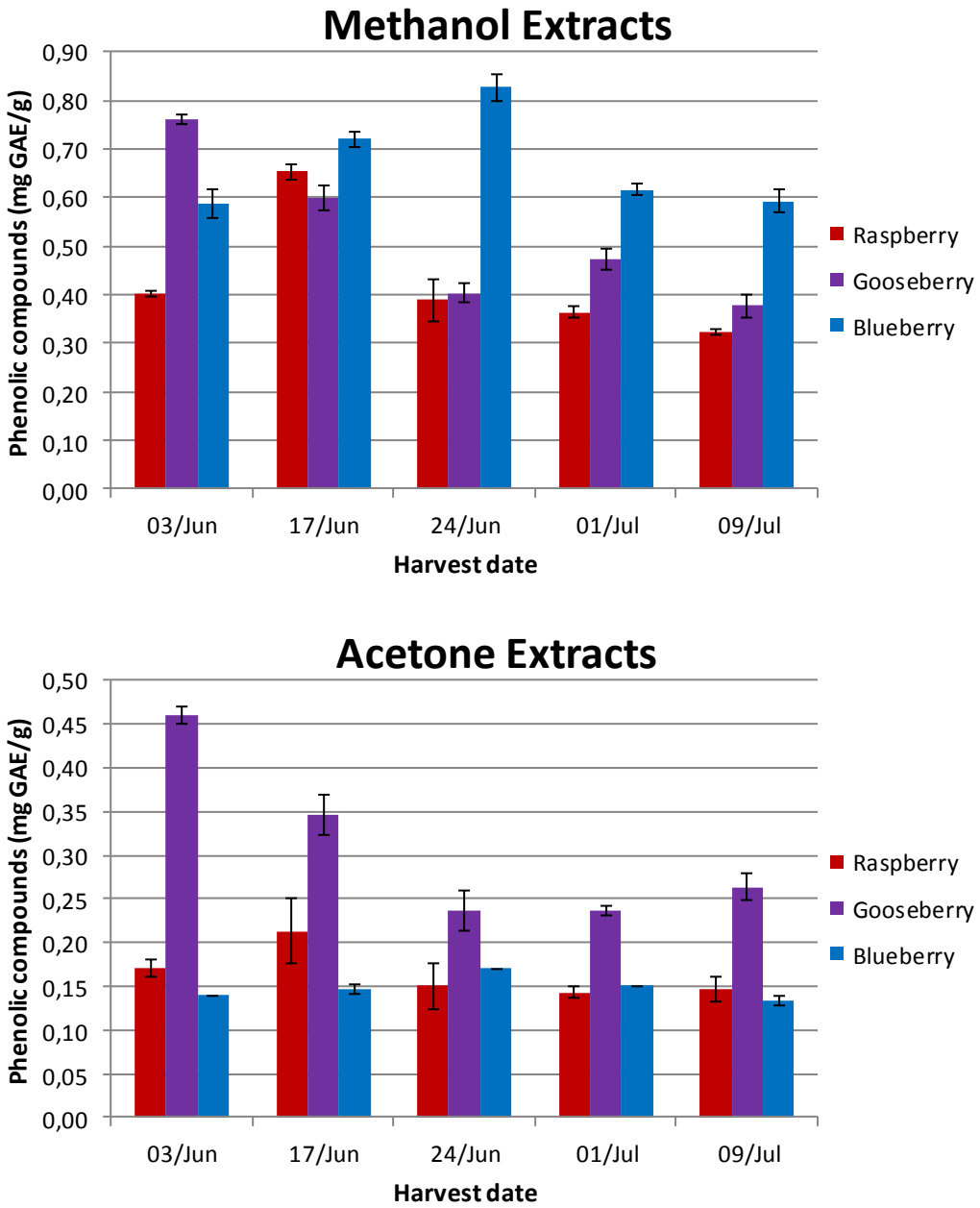

Fig. 1. Variation along maturation of the total phenolic compounds in the methanol and acetone extracts

The contents of phenolic compounds obtained in each extract for the different fruits at complete maturation (9/Jul) are shown in Fig. 2. The total of phenolic compounds for each fruit was obtained by summing the phenolic compounds on both extracts. Regarding the methanol extract, the higher amount of phenolic compounds was obtained for blueberry (0.59 mg/g GAE), followed by gooseberry (0.38 mg/g GAE) and raspberry (0.32 mg/g GAE). Otherwise, in the acetone extract, the gooseberry contained a higher amount of phenolic compounds (0.26 mg/g GAE). For all the studied fruits, the phenolic compounds were preferentially recovered in the methanol extract when compared with the acetone extract, representing 59-82 % of the total quantified. The total phenols present in each fruit showed a higher value for blueberry, reaching 0.73 mg/g, of which 82 % were quantified in the methanol extract. Also Spagolla et al. (2009) quantified a higher amount of phenolic compounds in the methanol extract also for the

blueberry in similar conditions of extraction and analysis. Gooseberry and raspberry exhibited 0.64 and 0.32 mg/g GAE, respectively, in which the methanol extracts represented respectively 59 % and 68 %. These values are lower than those described in the bibliography by Kuskoski et al. (2006) for grape, mulberry and strawberry (1.17, 1.82 and 1.32 mg/g GAE, respectively) and by Sun et al. (2002) for grape an strawberry (1.82 and 1.48 mg/g GAE).

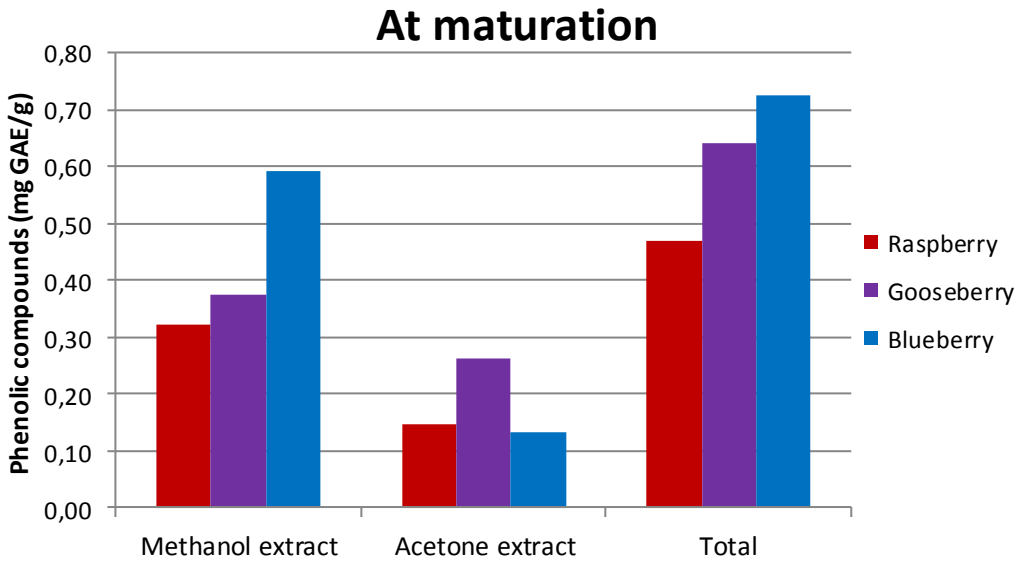

Fig. 2. Phenolic compounds at complete maturation

Fig. 3 presents the evolution of antioxidant activity during maturation, determined by ABTS and DPPH methods. In all cases it was observed that the quantification of the antioxidant activity by the DPPH method was much inferior to that quantified by the ABTS method, between 15 % and 19 % for raspberry, between 17 % and 21 % for gooseberry and between 9 % and 12 % in the blueberry. Regarding the evolution along maturation, while raspberry and gooseberry show a decrease in the antioxidant activity, the blueberry shows a marked increase. In the case of raspberry, the values of the antioxidant activity at the end of maturation represented 98 % and 72 % of those observed on the initiation of maturation for the ABTS and DPPH methods, respectively, indicating that the higher decrease was quantified by the DPPH method. For gooseberry, the decrease in the antioxidant activity during maturation was 45 % and 50 %, respectively for ABTS and DPPH methods. On the contrary, for blueberry the increase was 153 % and 124 % in relation to the activity initially quantified, also for both methods.

Fig. 4 shows the evolution of the content in tannins and anthocyanins throughout the maturation for the three fruits studied. The total amount of tannins in raspberry varied between 0.11 and 1.17 mg/g, in gooseberry between 0.18 and 1.19 mg/g and in blueberry between 0.96 and 1.38 mg/g. With respect to the evolution along maturation, while in raspberry the total tannins decrease markedly, in gooseberry they increase significantly and in blueberry they also increase. Furthermore, in general the blueberry shows higher amounts of tannins in comparison with the other fruits. At the end of ripening, the fruits showed differences, being the higher amount quantified again in blueberry (1.38 mg/g) in comparison with gooseberry (1.11 mg/g) and raspberry (0.15 mg/g).

Regarding the evolution of anthocyanins along the ripening of fruits (Fig. 4), it was found that blueberry always got the highest values (from 0.52 to 0.78 mg/g EMvG), followed by raspberry (from 0.13 to 0.40 mg/g EMvG) and by gooseberry (from 0.02 to 0.19 mg/g EMvG). At the end of ripening, the blueberry contained a significantly higher value of total anthocyanins (0.78 mg/g MvGE) when compared with raspberry and gooseberry (0.13 mg/g McGE, in both cases). The value found on this study for blueberry was similar to that described in literature by Pertuzatti et al. (2007) (0.81 mg/g MvGE) but higher than that obtained by Rocha (2009) (0.58 mg/g MvGE), in both cases also for blueberries.

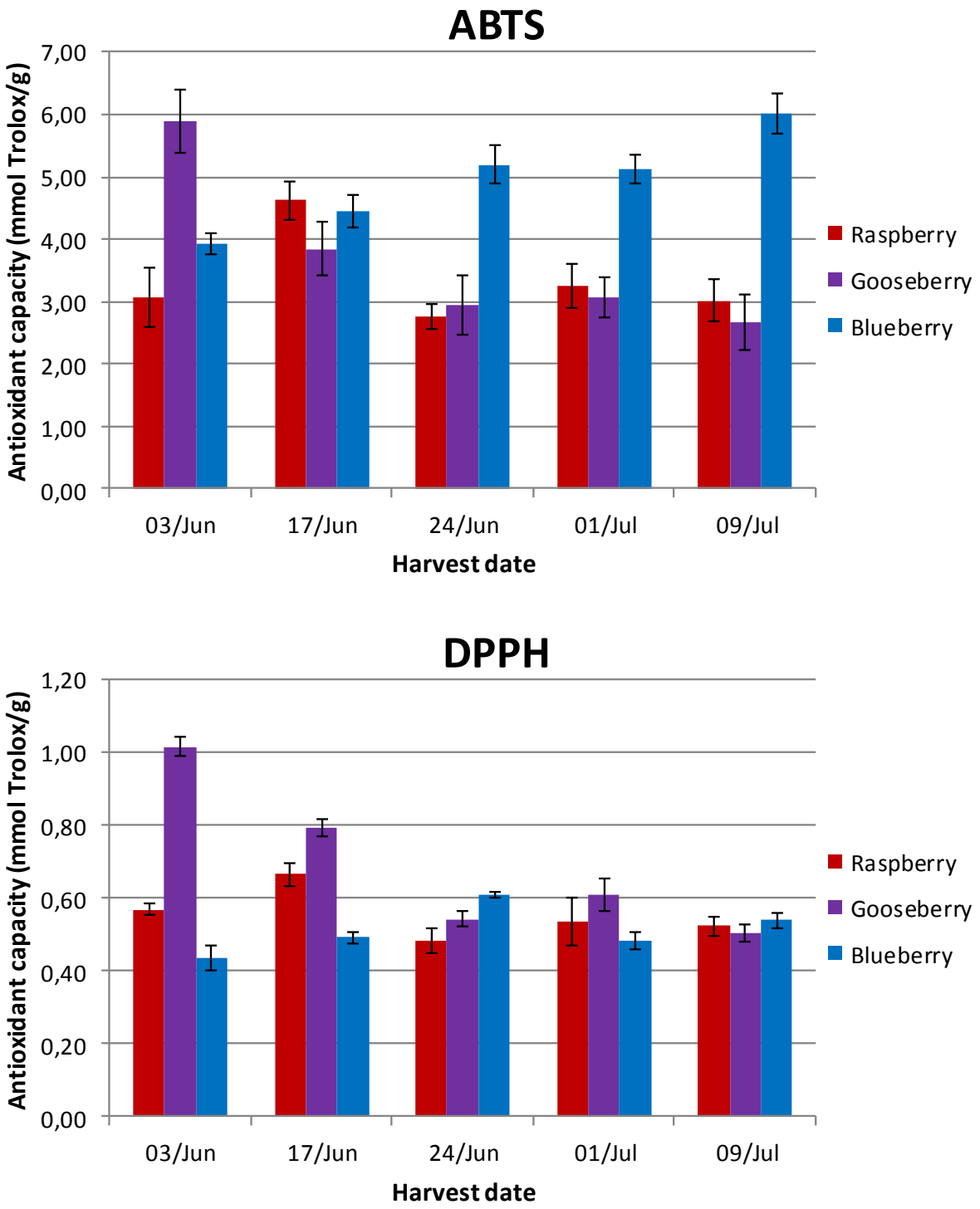

Fig. 3. Variation along maturation of the antioxidant capacity determined by the ABTS and DPPH methods

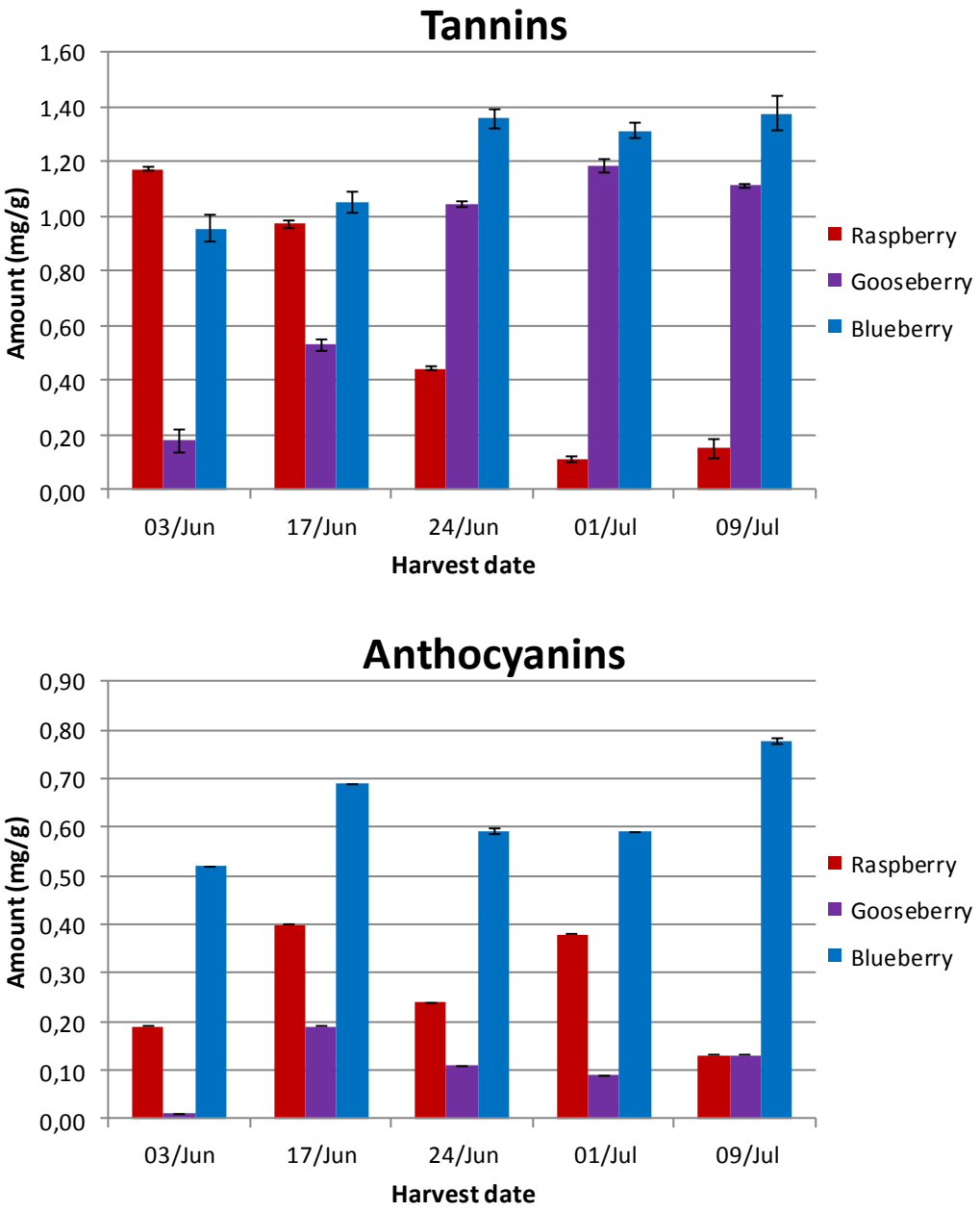

Fig. 4. Variation along maturation of the total tannins and total anthocyanins

The anthocyanins may be present in natural products in the monomer, polymer and co-pigmented forms. Table 1 shows the evolution along maturation in the three fruits studied of the different forms of anthocyanins. In general, there was an increase in the relative percentage of co-pigmented forms and a decrease in monomeric forms. The anthocyanins in raspberry at complete maturation were preferentially in the monomeric form (67 %), while the co-pigmented and polymeric forms represented 18 and 15 %, respectively. In gooseberry at the beginning of maturation the monomeric forms accounted for 93 % of all anthocyanins, decreasing to 16 % at the end of maturation, when the co-pigmented forms became predominant accounting for 67 % of the total amount. In blueberry also occurred a decrease of the monomeric forms, although less pronounced than in other fruits, namely from 94 % at start to 48 % at the end of maturation. At complete maturation the co-pigmented forms represented 46 % of the anthocyanins present, being this amount very similar to that of the monomeric forms.

Table 1. Variation along maturation of the percentage of monomeric, polymeric and co-pigmented anthocyanins

Anthocyanin forms	**Amount (%)**				
	03/Jun	17/Jun	24/Jun	01/Jul	09/Jul
	Raspberry				
Monomeric	63	50	49	34	18
Polymeric	10	8	7	4	15
Co-pigmented	27	42	44	62	67
	Gooseberry				
Monomeric	93	64	48	15	16
Polymeric	2	5	6	12	17
Co-pigmented	5	31	47	73	67
	Blueberry				
Monomeric	94	62	68	59	48
Polymeric	6	15	13	11	6
Co-pigmented	0	24	19	31	46

Fig. 5 shows the anthocyanin profiles obtained by HPLC for the fruits studied, raspberry, gooseberry and blueberry, which allowed identifying some monomeric anthocyanins present that are reported in Table 2.

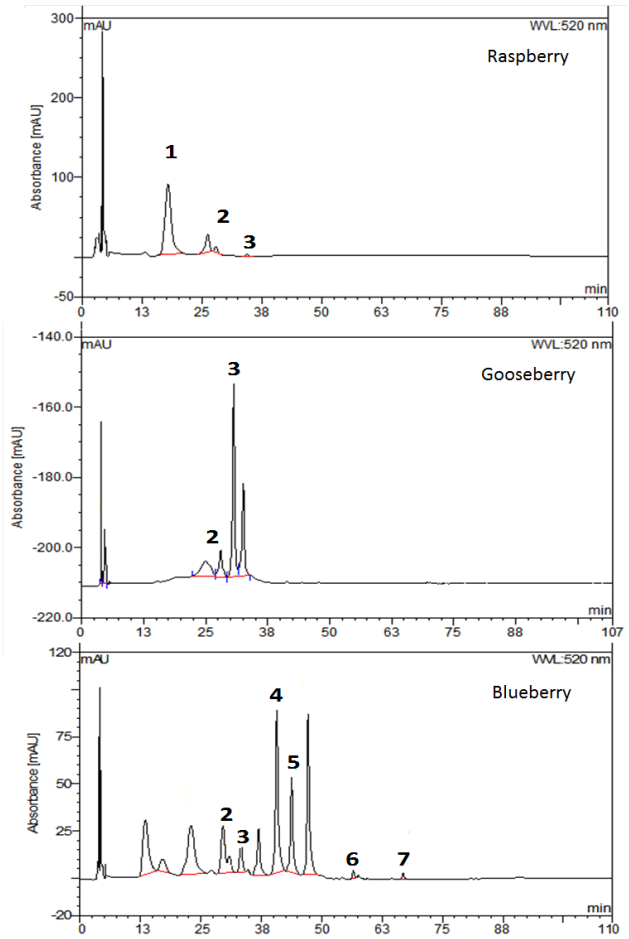

Fig. 5. Chromatographic profiles of the monomeric anthocyanins quantified in the three fruits at complete maturation. (1) delphinidin 3-glucoside (2) cyanidin 3-glucoside (3) petunidin 3-glucoside (4) peonidin 3-glucoside (5) malvidin 3-glucoside (6) petunidin 3-acetylglucoside (7) malvidin 3-acetylglucoside

Table 2. Monomeric anthocyanins composition at complete maturation

Monomeric anthocyanins	Raspberry	Gooseberry	Blueberry
Cyanidin 3-glucoside	3.95	4.84	17.85
Delphinidin 3-glucoside	106.27	n.d.	n.d.
Malvidin 3-acetylglucoside	n.d.	n.d.	1.07
Malvidin 3-glucoside	n.d.	n.d.	24.96
Peonidin 3-glucoside	n.d.	n.d.	45.25
Petunidin 3-acetylglucoside	n.d.	n.d.	1.41
Petunidin 3-glucoside	1.36	21.94	8.40

Results are expressed as μg/g MvGE. n.d. = not detected.

From the results obtained it was possible to identify six anthocyanins in blueberry, three in raspberry and two in gooseberry. The total amount of monomeric anthocyanins quantified in raspberry, gooseberry and blueberry was 111.6, 26.7 and 98.8 μg/g MvGE, respectively. In raspberry, the delphinidin 3-glucoside was present in higher amount (106.27 μg/g MvGE), which was in accordance with the findings of Obón et al. (2012) and Goiffon et al. (1999). The gooseberry was composed of 21.94 μg/g MvGE of petunidin 3-glucoside and 4.84 μg/g MvGE of cyanidin 3-glucoside. The presence of these anthocyanins was previously described in gooseberry (Goiffon et al., 1999; Wu and Prior, 2005). The blueberry was mainly composed by peonidin 3-glucoside (45.25 μg/g MvGE), malvidin 3-glucoside (24.96 μg/g MvGE) and cyanidin 3-glucoside (17.85 μg/g MvGE). The remaining quantified anthocyanins accounted for 10.9 μg/g MvGE. The presence of these anthocyanins was previously described in literature (Obón et al., 2011; Del Rio et al., 2010; Versari et al., 1997).

With regard to phenolic acids present in the fruits studied, they were analysed using HPLC by using two wavelengths for the detection (280 and 325 nm). Figure 6 shows the profiles obtained for the different phenolic acids detected in raspberry, gooseberry and blueberry, respectively, and the different compounds identified are shown in Table 3. The raspberry (52.10 μg/g GAE) and gooseberry (42.58 μg/g GAE) contained higher amounts of phenolic acids when compared with blueberry (5.45 μg/g GAE). In case of raspberry and gooseberry was possible to identify three hydroxybenzoic acids and four and five hydroxycinnamic acids, respectively. For blueberry was possible to identify four hydroxybenzoic acids and one hydroxycinnamic acid. The total of hydroxybenzoic acids present in raspberry accounted for 14.48 μg/g GAE, of which vanilic acid represented 75 %. The hydroxycinnamic acids represented 37.62 μg/g GAE, of which 79 % were ferulic acid. Gooseberry contained 10.54 μg/g GAE, of hydroxybenzoic and 32.04 μg/g GAE of hydroxycinnamic acids. The quantification of phenolic acids (hydroxycinnamic acids) in blueberry has been reported by Castrejón et al. (2008). Chiang et al. (2013) reported some phenolic acids in gooseberry, namely the hydroxycinnamic acid p-coumaric. Also Gevrenova et al. (2013) reported some hydroxycinnamic acids in raspberry (p-coumaric and ferulic).

Table 3. Phenolic acids (Hydroxybenzoic and Hydroxycinnamic) present at complete maturation

Phenolic acids	Raspberry	Gooseberry	Blueberry
Hydroxybenzoic acids			
gallic	n.d.	n.d.	1.27
p-hydroxybenzoic	n.d.	n.d.	0.54
protocatechuic	1.10	7.98	n.d.
syringic	2.44	1.29	0.91
vanilic	10.94	1.27	0.58
Hydroxycinnamic acids			
caftaric	1.06	3.58	n.d.
chlorogenic	3.07	9.58	2.15
ferulic	27.26	7.97	n.d.
p-coumaric	6.23	2.10	n.d.
p-hydroxycinnamic	n.d.	8.81	n.d.

Results are expressed as μg/g GAE. n.d. = not detected.

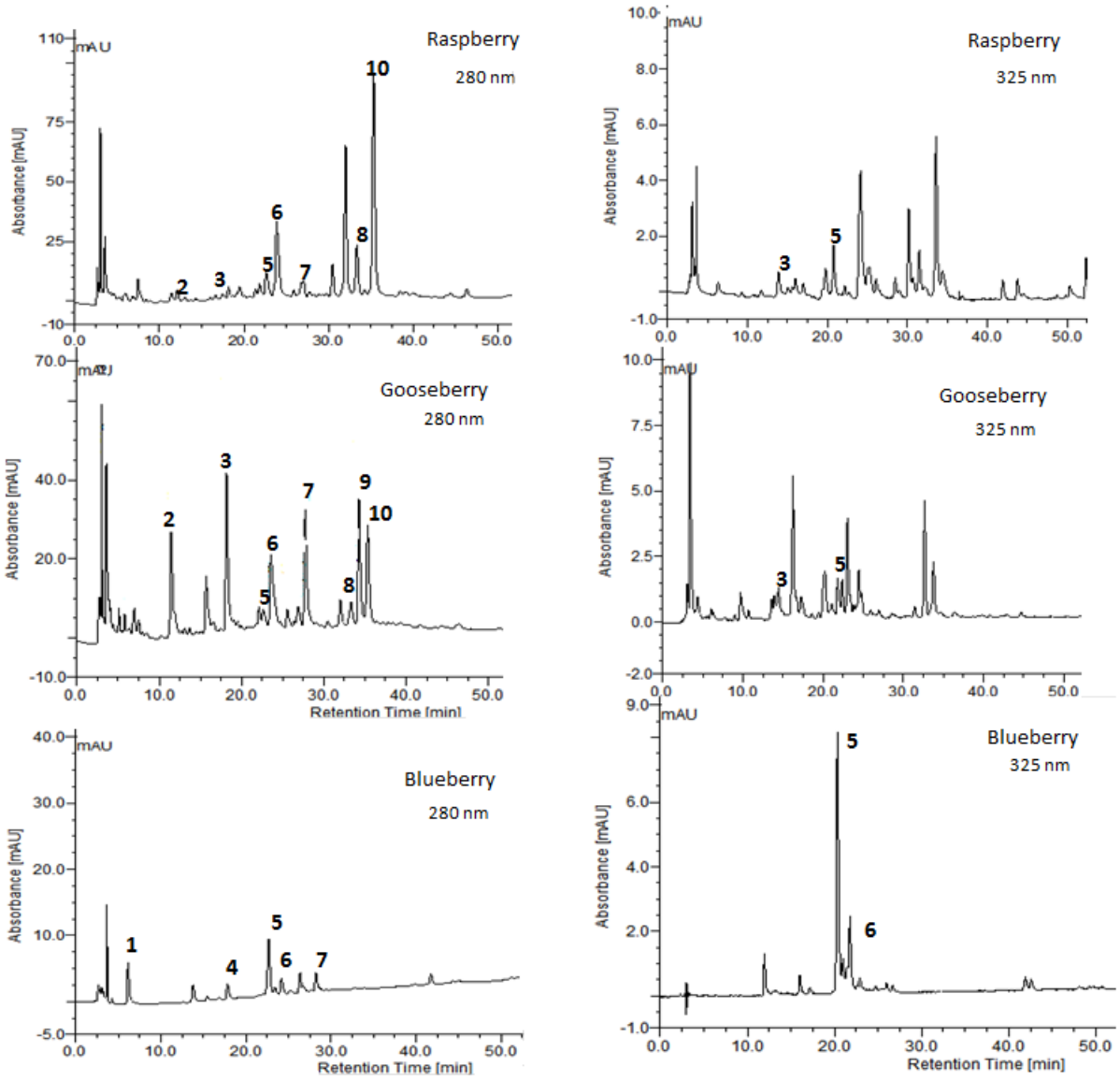

Fig. 6. Chromatographic profiles (280 and 325 nm) of the phenolic acids quantified in the three fruits at complete maturation. (1) gallic (2) protocatechuic (3) caftaric (4) *p*-hydroxybenzoic (5) chlorogenic (6) vanilic (7) syringic (8) *p*-coumaric (9) ferulic

All of the three studied fruits have important quantities of several phenolic compounds with proved health benefits. The flavonoids and phenolic acids present in raspberry, besides their antioxidant activity, have antimutagenic, anticarcinogenic, cytotoxic, and antimicrobial properties (Badjakov et al., 2008; Durgo et al., 2012; Kähkönen et al., 2012; Thiem, 2003). Besides Han et al. (2012) reported that p-coumaric acid exhibited anticoagulant and antiplateled activities, and this hydroxycinnamic acid was quantified in raspberry and gooseberry. Prince et al (2011) reported that the pretreatment with vanillic acid showed significant protective effects on cardiac troponins, lipid peroxidation, antioxidant system, electrocardiogram and expressions of interleukin-1β, interleukin-6 and tumor necrosis factor-α gene in the heart of isoproterenol induced cardiotoxic rats. This yydroxybenzoic acid was quantified in a very high amount in raspberry and also, although less, in blueberry and gooseberry.

The content of phenolics in berries is affected by the degree of maturity at harvest, besides other factors, and therefore the degree of ripening is very important for the determination of the product biological activities (Castrejón et al., 2008; Zadernowski et al., 2005).

Conclusions

The total phenolic compounds of all fruits decreased along maturation in both extracts, and the amount quantified in the methanol extract was always higher than in the acetone extract.
Regarding the antioxidant capacity, the values obtained for the three fruits were similar when determined by DPPH method, whereas by the ABTS method was observed a difference in blueberry, which showed higher antioxidant activity.
The amounts of totals tannins and anthocyanins quantified in blueberries and in blackcurrants increased throughout maturation. On the contrary, raspberries had lower levels of these compounds at the end of ripening, compared with the beginning of maturation.
At the end of maturation, the blueberry contained higher amounts of phenolic compounds, total tannins and total anthocyanins than raspberry and gooseberry.
Throughout maturation it was observed for the three fruits a decrease in the relative percentage of monomeric forms of anthocyanins and an increase in the co-pigmented forms.
HPLC analysis allowed to identify the presence of monomeric anthocyanins and phenolic acids (benzoic and cinnamic) in raspberry, gooseberry and the blueberry.

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